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Pharmacological and Biochemical Studies of Cytotoxicity of *Clostridium novyi* Type A Alpha-Toxin

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The actions of apparently homogeneous alpha-toxin from *Clostridium novyi* type A were studied in order to develop an in vitro system which closely mimicks its in vivo effects and to search for the mode of poisoning. Time to death (by intravenous injection of mice) was inversely related to dose, with a detection limit of about 200 ng/kg of body weight at 100 h. Injections of 2.5 ng or more into the rat paw led to a slowly (maximum after about 30 h) developing, dose-dependent edema which was useful as a quantitative in vivo assay based on volumetry. Vascular leakage was due to gap formation between endothelial cells. Similarly, endothelial cells cultured from pig pulmonary artery lost their "cobblestone" arrangement after a dose-dependent lag period of some hours after poisoning. The morphological changes were accompanied by depression of uptake or incorporation of [^{3}H]uridine. A quantitative in vitro assay was established on the inhibition of [^{3}H]uridine incorporation. As in animals, the action of alpha-toxin started with a few nanograms per milliliter and proceeded slowly for at least 1 day but became resistant to antitoxin within 2 h of exposure. The toxin action is not limited to endothelial cells, since chicken embryonic cells, a mouse fibroblast line (L-929), and a rat phaeochromocytoma line (PC-12) behaved similarly. Alpha-toxin was found to differ from other bacterial toxins investigated whose modes of action are already known.

Clostridium novyi is classified into four types designated A, B, C, and D, according to the respective soluble antigens. Only types A and B synthesize the lethal alpha-toxin (19). Type A is involved in gas gangrene infections in man and animals (1). Type B causes infectious necrotic hepatitis (black disease) in sheep and other animals (4).

Culture filtrates of *C. novyi* type B given intradermally induce vascular permeability which starts slowly and lasts for days (10) and which is due to formation of gaps between the endothelial cells of small and medium-sized venules (8). Culture filtrates of *C. novyi* types A and B also cause cytopathic alterations in cultures of chicken embryonic cells (CEC), MK or BHK cells (21), and human embryonic lung fibroblasts (22).

C. novyi type A alpha-toxin is the only factor of the culture filtrate that is both lethal and edematizing in vivo (13, 14). Nothing is known about the mode of action of this unusually potent and long-acting toxin. Even quantitative tests which are prerequisites for studies of that kind are lacking. A noninvasive in vivo test should quantify the course and amount of extravasation. An in vitro test should mimick the in vivo actions as closely as possible, i.e., formation of interendothelial gaps, and simultaneously allow monitoring of the cytopathic effect by a quantitative biochemical method.

We show here that the former requirement can be met by volumetry of the rat paw edema, and the latter by examining the effect on cultured endothelial cells. It is evident that alpha-toxin differs from other bacterial toxins investigated whose modes of action are already known.

MATERIALS AND METHODS

Reagents. Medium 199, Dulbecco modified Eagle medium (DMEM), compound 48/80, poly-L-lysine, high-molecular-weight marker (SDS-6H), and bovine serum albumin were obtained from Sigma, Deisenhofen, Federal Republic of Germany. Fetal calf serum and ATP bioluminescence reagent CLS were from Boehringer GmbH, Mannheim, Federal Republic of Germany; calf serum and horse serum were from Biochrom, West Berlin; penicillin G and streptomycin were from Serva, Heidelberg, Federal Republic of Germany; and Proteose Peptone and yeast extract were from Difco Laboratories, Detroit, Mich. The following radiochemicals were purchased from Amersham Buchler, Braunschweig, Federal Republic of Germany: [5,6-^{3}H]uridine (40 Ci/mmol), [6-^{3}H]thymidine (22 Ci/mmol), L-[U-^{14}C]leucine (330 mCi/mmol), L-[^{35}S]methionine (>800 Ci/mmol, translation grade), L-[2,5,6-^{3}H]norepinephrine, and [α-^{32}P]NAD (>800 Ci/mmol) were from NEN Chemicals GmbH, Dreieich, Federal Republic of Germany. Diphtheria toxin was a gift from D. M. Gill, Harvard University, Cambridge, Mass., and botulinum C_2-toxin was from K. Aktories, Rudolf-Buchheim Institute of Pharmacology. Horse antiserum against a concentrated culture filtrate preparation of *C. novyi* was obtained from Behringwerke, Marburg, Federal Republic of Germany.

Toxin production and purification. *C. novyi* type A (strain 19402) was cultured anaerobically for 6 days at 37°C in 0.8-liter portions of a medium containing 2% Proteose Peptone, 1% yeast extract, 1% glucose, 0.5% $CaCO_3$, and 0.05% sodium thioglycolate. Five cultures were then combined and cleared by centrifugation. For purification of alpha-toxin, we modified a published procedure (13). Briefly, the supernatant was treated with 38 g of ammonium sulfate per 100 ml. After 2 h at room temperature, the precipitate was spun down and the pellet was redissolved in a mixture containing 50 mM Tris hydrochloride and 0.5 mM EDTA (pH 7.9). In this way, 2.5×10^7 50% lethal dose (LD_{50}) units (LD_{50} of 2.5 μg of protein per kg of body weight) was obtained and set as 100%. The dialyzed solution was applied to a DEAE-cellulose

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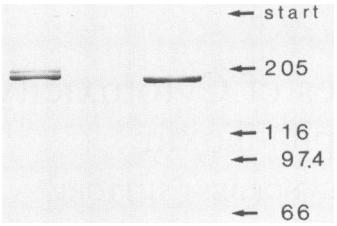

FIG. 1. Purity and size of purified *C. novyi* alpha-toxin in SDS-polyacrylamide gel electrophoresis. Proteins were stained with Coomassie blue. Lanes: left, 1 μg of alpha-toxin, not reduced; right, 1 μg of alpha-toxin, reduced. The standards (kilodaltons) were myosin (205), β-galactosidase (116), phosphorylase B (97), and bovine serum albumin (66).

column (2.5 × 16 cm). The bound toxin was eluted with a NaCl gradient at about 80 mM, resulting in 1.86×10^7 LD_{50} units (LD_{50} of 0.53 μg/kg). After dialysis, the toxin was adsorbed on an SP-Sephadex column (2.5 × 10 cm) at pH 5.7 (20 mM sodium phosphate) and desorbed with a NaCl gradient at about 150 mM. The toxin (6.3×10^6 LD_{50} units with a LD_{50} of 0.17 μg/kg) was concentrated in a Centriflo membrane cone (CF25; Amicon Corp., Lexington, Mass.). Minor impurities were removed by a final passage through a Sephacryl S 300 column (1.6 × 75 cm) with a mixture of 50 mM sodium phosphate, 150 mM NaCl, and 1 mM EDTA (pH 7.5). Final yield was 2.6×10^6 LD_{50} units (i.e., 13% with respect to the ammonium sulfate precipitate) in 16 mg of protein (LD_{50} of 200 ng/kg). There was no evidence that another lethal and edematizing constituent had been separated from alpha-toxin by any of the purification steps. No search was made for modifiers of toxicity which might accompany alpha-toxin.

When the purified toxin was heated in sodium dodecyl sulfate (SDS) without a reducing agent, SDS-polyacrylamide gel electrophoresis (16) revealed a faster main band immediately followed by a smaller one. Reduced alpha-toxin formed a single band, indicating the absence of disulfide-linked chains (Fig. 1). Toxin portions (280 μg/ml in 150 mM NaCl–50 mM sodium phosphate (pH 7.4)–1 mM EDTA) were stable for at least 15 months when frozen in liquid nitrogen and stored at −20°C. Further dilutions contained 0.1% bovine serum albumin as a protective colloid.

Animal experiments. (i) General toxicity. Toxicity in mice was assayed by intravenous injection of toxin dilutions (10 μl per g of body weight). Time to death was recorded for 100 h (6). The LD_{50} was calculated for the 100-h observation time. The plot of log time to death versus dose was linear between 2 and 100 h.

(ii) Local toxicity. The rat paw edema was monitored with a volumetric device (27) which consists of a 2-ml pipette welded with a short vertical glass cylinder at an angle of approximately 5°. The cylinder was filled with aqueous 20% isopropanol up to a mark. Toxin dilutions, or diluent without toxin (50 μl), were injected into the plantar tissue of the right hind paws of anesthetized (1.5 g of urethane per kg of body weight, subcutaneously) rats (Wistar, 250 to 300 g). At given times the injected paw and the contralateral controls were dipped into the glass cylinder up to a mark distal to their tarsal joints. The rise of the solution in the pipette indicated the volume of the paw. Vascular permeability was made visible by injections of alpha-toxin (between 10 ng and 1 pg in 50 μl) into the previously clipped and shaven abdominal skin of rats. After 20 h the rat was anesthetized intraperitoneally with 1 g of urethane per kg. Then (8) filtered carbon ink (1 ml/kg) with a mean particle size of 20 nm (C11/1431a, shellac-free; Pelikan, Hannover, Federal Republic of Germany) or (10) Evans blue (1 ml/kg, 1% solution) was injected intravenously. After 30 (with Evans blue) or 60 (with carbon ink) min, the rats were killed. The excised abdominal skin was stretched inside out on a small board and examined macroscopically. The carbon-labeled skin was pinned on a dental-wax plate, fixed in Formalin, cleared in glycerol, and examined by light microscopy from both sides.

Cell culture studies. (i) Cultivation of cells. All cultures were seeded initially on large plates (3.5- or 10-cm diameter) or in T75 flasks (endothelial cells), grown to near confluency, and then dispensed and subcultured for the experiments on multiwell plates (24 wells, 2-cm^2 area, 1-ml assay volume). All experiments were performed in triplicate. The cultures were regularly examined for cytopathic effects by phase-contrast microscopy. Viability of all types of cells was assessed by trypan blue dye exclusion.

(ii) Primary cultures of endothelial cells. Cultures were prepared (26) from pig pulmonary artery and grown in medium 199 containing 20% fetal calf serum, 50 U of penicillin G per ml, and 50 μg of streptomycin per ml. Experiments were carried out with confluent monolayers in passages 2 through 5. Primary cultures from chopped and trypsinized 10- to 12-day-old CEC were kept in DMEM with 10% calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). After 3 days they were subcultured in multiwell plates at a density of 10^5 cells per well by using DMEM with 5% calf serum. L-929 mouse fibroblasts (kindly supplied by Battelle Institute, Frankfurt, Federal Republic of Germany) were grown to confluence in DMEM with 10% calf serum and seeded on multiwell plates at a density of 5×10^4 cells per well. Rat phaeochromocytoma (PC-12) cells were plated in DMEM with 10% horse serum and 5% calf serum on poly-L-lysine-coated plates (3).

(iii) Inhibition of synthesis of RNA or DNA. The cells were exposed to toxin for 24 h if not otherwise stated. Endothelial cells were then washed with prewarmed medium 199, buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) and were labeled with 0.4 μCi of [^{3}H]uridine or 0.5 μCi of [^{3}H]thymidine per well in 0.5 ml of medium for 1 h at 37°C. L929, PC-12, and CEC cultures were washed and labeled in DMEM buffered with 20 mM HEPES (pH 7.4). The labeling medium was discarded, and the wells were washed twice with ice-cold Hanks balanced salt solution. Acid-soluble radioactivity was extracted by two changes of 0.5 ml of ice-cold 5% trichloroacetic acid and then measured. The residues were solubilized with 0.5 ml of 2% SDS for 30 min at room temperature. A sample (100 μl) of the solution was taken for protein determination (17). Acid-insoluble radioactivity was measured in the remaining 400 μl.

(iv) Inhibition of protein synthesis. The cell cultures were washed once and incubated for 1 h at 37°C in RPMI 1640 without leucine and isoleucine (Seromed, München, Federal Republic of Germany) and were buffered with HEPES (10 mM) and $NaHCO_3$ (5 mM). Then they were exposed to 0.1 μCi of [^{14}C]leucine for 1 h in 0.5 ml of the same medium. The wells were then processed as described above for nucleoside uptake and incorporation.

Tests for membrane damage. ATP was measured in endothelial cell cultures after 24 h of incubation with or without toxin. The cell layer was washed three times with ice-cold Hanks balanced salt solution without Ca^{2+} and Mg^{2+}, extracted with 0.4 N perchloric acid, and centrifuged. The supernatant was neutralized with 0.4 N KOH and brought to

pH 8.5 by the addition of 4 volumes of 50 mM glycine buffer. The pellet was dissolved in 2% SDS for protein determination (17). ATP levels were determined (9) in the diluted (phosphate-buffered saline, pH 7.4) solution by using the ATP bioluminescence CLS reagent. Release of lactate dehydrogenase from endothelial cells, and total lactate dehydrogenase activity was measured photometrically (25). [^{3}H]uridine leakage from CEC was measured as described previously (18). Histamine release from rat peritoneal mast cells was assayed (7) with compound 48/80 as a reference and with an exposure time of 30 min. Catecholamine release was tested with 5 μg of alpha-toxin per ml on washed rabbit brain homogenate preloaded with [^{3}H]norepinephrine (11). Horse, cattle, rabbit, and human erythrocytes were assessed for hemolysis and K^+ loss (12). After incubation of a 0.5% suspension of erythrocytes with 1 μg of alpha-toxin per ml for 30 min at 37°C, the suspension was placed on ice for another hour to search for hot-cold lysis. Intracellular potassium content in CEC and endothelial cells was measured after 24 h of incubation with 500 ng of alpha-toxin per ml or after 2 h of exposure to 5 μg/ml. Cell monolayers were washed with K^+-free phosphate-buffered saline. Residual K^+ was extracted by the addition of 1 ml of trichloroacetic acid per well, evaluated by flame photometry, and referred to cell protein (17).

Action of alpha-toxin in cell-free systems. ADP ribosylation was studied (2) with or without 5 μg of alpha-toxin per ml in endothelial-cell lysates prepared by freezing and thawing. Botulinum C_2I-toxin (1 μg/ml) served as a positive control. Cell-free protein synthesis was measured (20) in nuclease-treated rabbit reticulocyte lysate fortified with creatine phosphate, creatine phosphokinase, hemin, and calf liver tRNA (Promega Biotec, Madison, Wis.) under the influence of alpha-toxin or diphtheria toxin (10 μg/ml). In some experiments alpha-toxin was pretreated with 5 mM dithiothreitol or equimolar trypsin for 60 min at room temperature prior to addition.

Casein and gelatin served as substrates in the search for proteolytic activity. Thromboplastin time was determined by using Thromborel reagent (Behring AG, Frankfurt, Federal Republic of Germany). Electrophoresis was performed in SDS-polyacrylamide gels (16). Protein was determined (17) with bovine serum albumin as a standard.

RESULTS

Action on animals. (i) General toxicity. There was a dose-dependent decrease in survival time of mice upon intravenous injection of alpha-toxin. LD_{50} for purified alpha-toxin was about 200 ng/kg at 100 h. Symptoms indicating a participation of the central nervous system were absent. Dissection revealed general extravasation of fluid into the interstitial tissue and into the abdominal and pleural cavities but without macroscopic signs of bleeding. The symptoms suggest that progressive hypovolemic shock was the cause of death. Alpha-toxin (2 μg) was completely neutralized by antitoxin (1 μl).

(ii) Local toxicity. Subcutaneous injection of alpha-toxin caused a massive local edema extending far beyond the injection site. The underlying local vascular permeability was measured by plethysmography of the injected rat paw. After a lag period of at least 1 h, an edema developed with a detection limit of 2.5 ng of alpha-toxin. The degree of the swelling depended on dose and time. The effect of 2.5 ng per paw was maximal after 24 to 48 h and then faded slowly (Fig. 2).

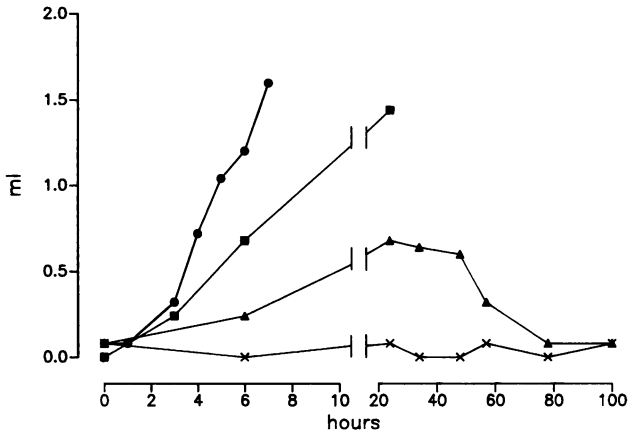

FIG. 2. Edema formation in the rat paw due to *C. novyi* alpha-toxin depending on concentration and time. Alpha-toxin (500 [●], 25 [■], or 2.5 [▲] ng) or solvent alone (50 μl [×]) was injected at zero time (one animal each). The ordinate gives the swelling (milliliters) of the injected paw measured against the volume of the contralateral paw. The animals with the most potent toxin concentrations were sacrificed because the edema had obstructed the venous blood flow.

To assess the mode of vascular leakage, we injected alpha-toxin intradermally and then applied Evans blue (10) or carbon ink (8) intravenously. Alpha-toxin (100 pg or more) promoted vascular reactions like those of *C. novyi* type B toxin (8, 10). Carbon ink depicted the boundaries of the endothelial cells at the basal membrane in the presence, but not in the absence, of 500 pg of alpha-toxin (results not shown). Apparently, formation of gaps between endothelial cells was the morphological basis for the severe vascular leakage with both toxins. Since cultured cells behaved in a similar manner, it appeared justified to substitute cell cultures for animals.

Action on cell cultures. (i) Morphological changes. On exposure to alpha-toxin, endothelial cells in culture lost their cobblestone structure after a dose-dependent latency of several hours. The cytopathic effect started with the formation of irregularly delineated gaps between endothelial cells, which remained attached to one another by slender filaments. This behavior has been defined as retraction (28). Finally the cells became round (Fig. 3). As shown by the dye exclusion test, poisoned cells remained viable for at least 48 h, although all cells had become spherical after 24 h. Fibroblasts (for instance, CEC) also tended toward rounding. Since their two-dimensional pattern is less ordered than that of endothelial cells, its disorganization by the toxins was more difficult to recognize. The detection limit for onset of morphological alterations was about 5 ng of alpha-toxin per ml with endothelial cells and about 15 ng/ml with CEC after 24 h of poisoning. The permanent cell lines tested (L-929 mouse fibroblasts and rat PC-12 cells) were much less toxin sensitive.

(ii) Biochemical changes. Especially in its early stage, the cytopathic effect of the toxin is difficult to quantify. To replace the morphological appraisal with a quantitative biochemical test, we evaluated the influence of alpha-toxin on the synthesis of RNA, DNA, and proteins. The toxin depressed equally well the appearance of radioactivity on exposure to [^{3}H]uridine in both the trichloroacetic acid-soluble (i.e., uptake) and the trichloroacetic acid-insoluble (i.e., incorporation) fractions of endothelial cells (Fig. 4).

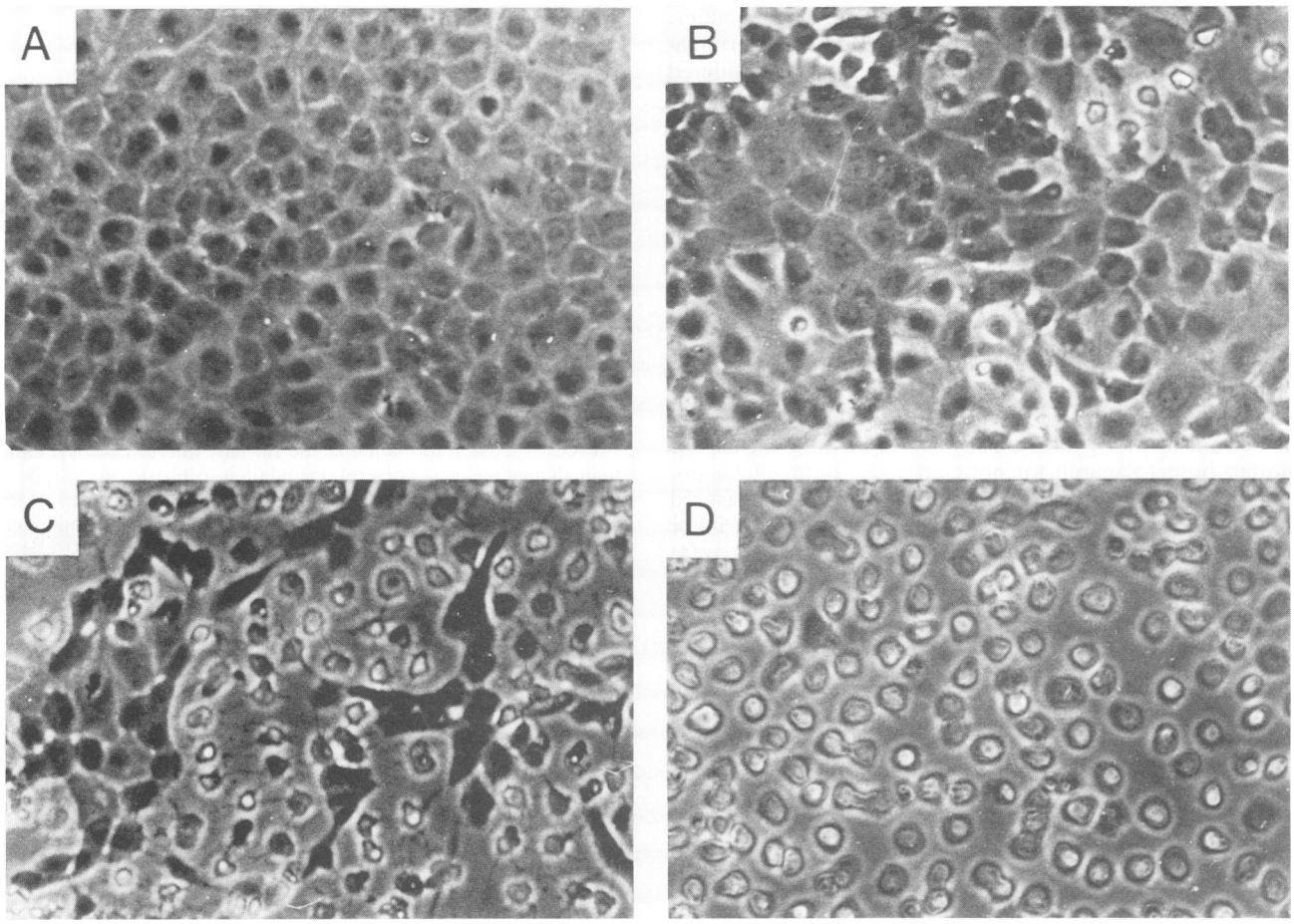

FIG. 3. Morphological signs of cytotoxicity in endothelial cells after 24 h of poisoning with alpha-toxin. Phase-contrast microscopy of control with typical cobblestone morphology (A); cells with 5 ng of toxin per ml, beginning retraction (B); cells with 50 ng of toxin per ml, 50% rounding with other cells retracted (C); cells with 500 ng of toxin per ml, complete rounding (D). The ratings for severity of damage were 0 (A), + (B), ++ (C), and +++ (D).

Cellular handling of thymidine and leucine was influenced similarly (not shown).

Depression of uridine incorporation was dose dependent with satisfactory standard deviations. It started with a few nanograms of toxin per milliliter and paralleled the development of the cytopathic effect. The plots did not deviate significantly whether incorporation was calculated per well or per milligram of protein, since the amount of protein per well decreased only moderately with the inhibition of RNA synthesis (Fig. 4).

The time course of alpha-toxin (200 ng/ml) action on uridine incorporation proceeded linearly over a 24-h period. The first cytopathic effects appeared after 4 h of latency, and cell rounding was 100% after 24 h (Fig. 5). Thus, the duration of incubation was set at 24 h in the standard assay. Sensitivity to antibodies decreased with the duration of previous exposure to toxin. Antitoxin (50 minimal neutralizing doses) had to be added within 1 h after intoxication (200 ng of alpha-toxin per ml) to prevent the toxin effect which would have otherwise developed during the next 23 h. Addition of antitoxin at the end of the latency (4 h after toxin addition) and later was ineffective (data not shown).

Sensitivity to alpha-toxin is not restricted to endothelial cells. The differences between the target cells were quantified by uridine incorporation. L-929 mouse fibroblasts and PC-12 cells were much less susceptible to alpha-toxin than were endothelial cells and CEC. However, biochemical and morphological changes ran approximately parallel in all cultures (Fig. 6).

(iii) Search for effects of alpha-toxin on membrane-dependent processes, protein synthesis, and ADP-ribosylation. Alpha-toxin lacked any pronounced effect on the membrane-related functions listed in Table 1. Particular attention should be given to the very modest decrease of ATP, which is a very sensitive parameter of cellular viability upon massive poisoning. CEC and endothelial cells also retained their intracellular potassium (given in micromoles per milligram of protein) after incubation with alpha-toxin (see Materials and Methods). We conclude that alpha-toxin does not damage cell membranes directly.

Depression of protein synthesis was assessed in reticulocyte lysate. As expected, diphtheria toxin (10 μg/ml) completely inhibited the translation of Brome mosaic virus RNA in the presence of 30 μM NAD, while alpha-toxin (10 μg/ml) had no effect on in vitro protein synthesis.

Alpha-toxin was compared with botulinum C_2-toxin with respect to ADP-ribosylation in endothelial cell lysates. Botulinum C_2-toxin ADP-ribosylated (2) a substrate of approximately 43 kilodaltons which comigrates with G-actin, whereas alpha-toxin did not label any substrate in the cell lysate (results not shown). Moreover, alpha-toxin neither cleaved casein or gelatin nor altered thromboplastin time in

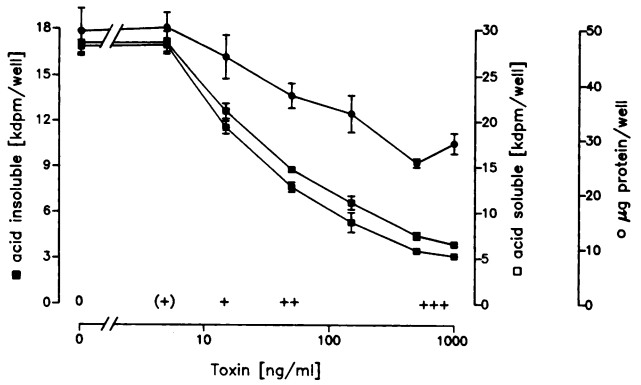

FIG. 4. Concentration-dependent inhibition of [^{3}H]uridine incorporation and uptake in endothelial cells paralleling morphological alterations. Bars represent the standard deviation of triplicate determinations. The experiments were repeated at least once with essentially the same results. Inhibition of uridine incorporation and uptake per well was statistically significant ($P < 0.01$) with toxin concentrations of ≥15 ng/ml. Symbols are explained in the legend to Fig. 3.

the Quick test. Thus, it is apparently not a broad-spectrum protease.

DISCUSSION

As stated 3 decades ago by Elder and Miles (10), alpha-toxin deserves study as a permeability factor, not only for its own sake but also as a unique tool for exploring the nature of pathological increases in capillary permeability, because "the much longer duration of the damage is extraordinary among all substances tested for their capillary permeability [and occurs] in doses that do not lead ultimately to necrosis." Cotran (8) observed the formation of intercellular gaps in the endothelium of small and medium-sized venules when they were exposed to *C. novyi* type B toxin in situ. He confirmed that the onset of permeability increase was delayed and that duration was extraordinarily prolonged. We have now introduced rat paw edema measurement as a volumetric method to monitor the change of permeability continuously in vivo. Alpha-toxin-induced gap formation might be caused by active cell contraction, as is generally accepted for inflammatory mediators (15), or by rearrangement of the cytoskeleton, as has been shown for ethchlorvynol (28, 29) and cytochalasin B and D (24). On the other hand, venous vasoconstriction might increase intravascular pressure and widen the pores between intact endothelial cells passively.

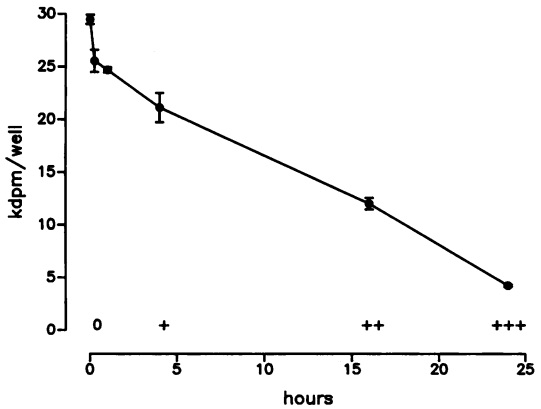

FIG. 5. Time-dependent inhibition of [^{3}H]uridine incorporation and morphological changes in endothelial cells. Cells were incubated with 200 ng of alpha-toxin per ml for the times given on the abscissa before all cells were labeled simultaneously as usual. The experiments were repeated at least once with essentially the same results. Symbols are explained in the legend to Fig. 3.

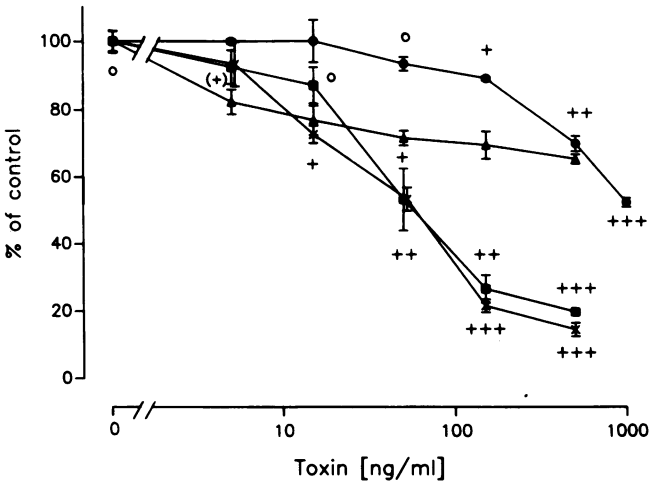

FIG. 6. Alpha-toxin sensitivity of different kinds of cultured cells. Cells were grown as described in Materials and Methods and poisoned for 24 h. Uridine incorporation (disintegrations per minute per well) in the toxin-free controls was 51,700 for L929 cells (●), 48,000 per PC12 cells (▲), 24,000 for endothelial cells (×), and 22,000 for CEC (■). For the sake of comparison, the control values were set at 100%. PC12 cells lost their processes after being poisoned with ≥15 ng of alpha-toxin per ml but did not display major additional cytopathic effects. Inhibition of uridine incorporation per well was significant ($P < 0.01$) in toxin concentrations of ≥15 ng/ml for endothelial cells, ≥50 ng/ml for CEC, ≥5 ng/ml for PC12 cells, and ≥500 ng/ml for L929 cells. The symbols for the cytopathic effect refer to the nearest graph. No symbol was attributed to the specific reaction of PC12 cells. The experiments were repeated at least once with essentially the same results. Symbols are explained in the legend to Fig. 3.

In the search for an in vitro system, we have reproduced the morphological changes of the vascular endothelium in culture. The isolated cells respond to alpha-toxin in a manner expected from the in vivo observations. The concentrations and incubation times of alpha-toxin required in vitro are in the range needed to elicit the rat paw edema. Despite these analogies, it remains to be shown whether the same mechanism underlies vascular leakage in vivo and retraction in vitro.

Depression of uridine uptake and incorporation was substituted for the observation of the morphological changes as a step towards quantification of alpha-toxin. Since decrease of RNA synthesis approximately parallels the degree of morphological alterations, the biochemical test probably depicts the events in the living animal. This conclusion is corroborated by the similar slow progression of poisoning in vivo and in vitro.

Whereas a correlation can be made between poisoned animals and equivalent responses in cell culture, the steps between the first toxin contact and the massive change in cell shape are still unknown. Inhibition of nucleic acid synthesis is a useful biochemical parameter but not necessarily the primary target of alpha-toxin action. Admittedly, depression of uptake or incorporation of uridine parallels the morpho-

TABLE 1. Failure of alpha-toxin to affect membrane-dependent processes

Cell type	Parameter	Reference or method	Highest toxin concn assessed (μg/ml)	Result
Erythrocytes from horse, cattle, rabbit, human	Hemolysis	12	1	No effect
	Loss of K^+	Flame photometry (12a)	1	No effect
Endothelial	Loss of LDH[a]	Photometry (25)	1	Less than 25% loss
	Trypan blue staining	0.04% Solution, 10-min incubation	0.5	No effect
	ATP content	Luciferase assay (9)	0.5	About 30% loss[b]
	K^+ content	Flame photometry (12a)	5	No effect
CEC	[^{3}H]uridine leakage	18	1.5	No effect
	K^+ content	Flame photometry (12a)	5	No effect
L929 mouse fibroblasts	Loss of LDH	Photometry (25)	1	No effect
PC12	Trypan blue staining	0.04% Solution, 10-min incubation	0.5	No effect
Rat peritoneal mast	Histamine release	7	10	No effect
Rabbit brain homogenate	[^{3}H]noradrenaline release	11	5	No effect

[a] LDH, Lactate dehydrogenase.

[b] ATP content (nanomoles per milligram of protein) without toxin was 58.7 ± 3.9. With 0.5 μg of alpha-toxin per ml for 24 h, it decreased to 41.3 ± 1.4 (mean ± standard deviation, $n = 3$).

logical changes in many cell types. However, cytochalasin B, whose primary action on the cytoskeleton is well accepted, inhibits nucleic acid synthesis as well (23). Accordingly, morphological alterations of anchorage-dependent mouse fibroblasts profoundly affect nucleic acid synthesis (5). We assume that the pronounced inhibition of uridine and thymidine uptake or incorporation, like other, more modest responses (Table 1), are secondary manifestations of a still unknown primary event.

Some pathogenic events due to other cytopathic bacterial toxins are irrelevant in the case of alpha-toxin. Unlike botulinum C_2 toxin, it is not an ADP-ribosyltransferase. Unlike clostridial neurotoxins, it does not inhibit noradrenaline release. Unlike diphtheria toxin, it does not inhibit protein synthesis in a cell-free system. Uptake of uridine into toxin-treated cells is decreased, probably because the turnover of the intracellular uridine pool is lowered. Early membrane damage is apparently not involved. For instance, cells displaying maximal cytopathic effects still retain 70% of their ATP and their full potassium content. Thus, alpha-toxin differs basically from bacterial cytolysins. By exclusion, one might assume that the toxin primarily attacks the cytoskeleton because of the changes of cell shape. Current work on endothelial cultures is attempting to reveal the earliest biochemical sequelae of toxin poisoning, among others, those linked with the cytoskeleton or with cell adhesion.

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